Pierre Fabre

INSTITUT DE RECHERCHE PIERRE FABRE

CENTRE D'IMMUNOLOGIE PIERRE FABRE

INDUSTRIAL BIOTECHNOLOGY DEPARTMENT

Ref : LCH/tp/2003-004

Object : patent application US09/673,288

To whom it may concern,

I, the undersigned Laurent Chevalet,

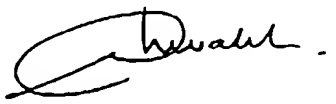
domiciled : 1241 route de Ferrières – (F) 74350 CUVAT,
presently working for the Pierre Fabre Group as Head of the Industrial Biotechnology Department of the Centre d'Immunologie based in Saint-Julien en Genevois, France,
having the following qualification : Engineer in Biotechnology, PhD in Microbial Genetics,

hereby state and certify that :

- any person skilled in the art performing an amplification reaction, using (i) the oligonucleotides Trp5 and Trp2 and (ii) E. coli chromosomal DNA as template, will generate a DNA fragment, hereby named fragment 1, having a sequence encompassing positions 1 to 503 of the sequence described in appendice 1.
- any person skilled in the art performing an amplification reaction, using (i) the oligonucleotides Trp3 and Trp4 and (ii) E. coli chromosomal DNA as template, will generate a DNA fragment, hereby named fragment 2, having a sequence encompassing positions 498 to 1335 of the sequence described in appendice 1.
- any person skilled in the art ligating the above mentioned fragments 1 and 2 will generate a DNA fragment having the sequence described in appendice 1.
- any person skilled in the art performing an amplification reaction, using (i) the oligonucleotides TrpR1 and TrpR2 and (ii) E. coli chromosomal DNA as template, will generate a DNA fragment, hereby named fragment 3, having a sequence encompassing positions 1 to 520 of the sequence described in appendice 2.
- any person skilled in the art performing an amplification reaction, using (i) the oligonucleotides TrpR3 and TrpR4 and (ii) E. coli chromosomal DNA as template, will generate a DNA fragment, hereby named fragment 4, having a sequence encompassing positions 508 to 843 of the sequence described in appendice 2.

- any person skilled in the art performing an amplification reaction, using (i) the oligonucleotides TrpR5 and TrpR6 and (ii) E. coli chromosomal DNA as template, will generate a DNA fragment, hereby named fragment 5, having a sequence encompassing positions 838 to 1468 of the sequence described in appendice 2.
- any person skilled in the art linking fragments 3 and 4 together and ligating them with fragment 5 will generate a DNA fragment having the sequence described in appendice 2.
- the two following publications : Deeley & Yanofsky (1981), and Gunsalus & Yanofsky (1980) cited in the above-mentioned US patent application provide evidence of the sequences described in appendices 1 and 2.

Made in Saint-Julien en Genevois, February 27th, 2003.



Appendice 1 : sequence used to inactivate the tryptophanase tnaA gene by insertion of a stop codon (construction of ICONE 100)

Nucleotides 1 to 275 : sequence of the E. coli tnaA promoter
Nucleotides 276 to 1335 : 5' part of the E . coli tnaA gene coding sequence
Nucleotides 276 to 278 : start codon of the E. coli tnaA gene
Nucleotides 495 to 503 : stop codon inserted in the E. coli tnaA gene followed by an XbaI restriction site

GTGTGACCTCAAAATGGTTCAATATTGACAACAAAATTGTCGATCACCGCCCTTGATTTGCCCTTCTGTAGCCAT
CACCAGAGCCAAACCGATTGATTCAATGTGTTCTATTTGTTTGCTATATCTTAATTTTGCCTTTTGCAAAGGTCA
TCTCTCGTTTATTTACTTGTTTTAGTAAATGATGGTGCTTGCATATATATCTGGCGAATTAATCGGTATAGCAGA
TGTAATATTCACAGGGATCACTGTAATTAAAATAAATGAAGGATTATGTA**ATG**GAAAACTTTAAACATCTCCCTG
AACCGTTCCGCATTCGTGTTATTGAGCCAGTAAAACGTACCACTCGCGCTTATCGTGAAGAGGCAATTATTAAAT
CCGGTATGAACCCGTTCCTGCTGGATAGCGAAGATGTTTTTATCGATTTACTGACCGACAGCGGCACCGGGGCGG
TGACGCAGAGCATGCAGGCTGCGATGATGCGCGGCGACGAAGCC**TAATCTAGA**CAGCGGCAGTCGTAGCTACTAT
GCGTTAGCCGAGTCAGTGAAAAATATCTTCGGTTATCAATACACCATTCCGACTCACCAGGGCCGTGGCGCAGAG
CAAATCTATATTCCGGTACTGATTAAAAAACGCGAGCAGGAAAAAGGCCTGGATCGCAGCAAAATGGTGGCGTTC
TCTAACTATTTCTTTGATACCACGCAGGGCCATAGCCAGATCAACGGCTGTACCGTGCGTAACGTCTATATCAAA
GAAGCCTTCGATACGGGCGTGCGTTACGACTTTAAAGGCAACTTTGACCTTGAGGGATTAGAACGCGGTATTGAA
GAAGTTGGTCCGAATAACGTGCCGTATATCGTTGCAACCATCACCAGTAACTCTGCAGGTGGTCAGCCGGTTTCA
CTGGCAAACTTAAAAGCGATGTACAGCATCGCGAAGAAATACGATATTCCGGTGGTAATGGACTCCGCGCGCTTT
GCTGAAAACGCCTATTTCATTAAGCAGCGTGAAGCAGAATACAAAGACTGGACCATCGAGCAGATCACCCGCGAA
ACCTACAAATATGCCGATATGCTGGCGATGTCCGCCAAGAAAGATGCGATGGTGCCGATGGGCGGCCTGCTGTGC
ATGAAAGACGACAGCTTCTTTGATGTGTACACCGAGTGCAGAACCCTTTGCGTGGTGCAGGAAGGCTTCCCGACA
TATGGCGGCCTAGAAGGCGGCGCGATGGAGCGTCTGGCGGTAGGTCTGTATGACGGCATGAATCTCGACTGGCTG
GCTTATCGTATCGCGCAGGTACAGTATCTGGTCGATGGTCTGGAAGAGATTGGCGTTGTC

Appendice 2 : sequence used to inactivate the tryptophanase tnaA gene by replacement with the trpR gene (construction of ICONE 200)

Nucleotides 1 to 510 : sequence of the E. coli tnaA promoter
Nucleotides 511 to 837 : sequence of the E. coli trpR gene
Nucleotides 511 to 513 and 835 to 837 respectively : start and stop codons of the E. coli trpR gene
Nucleotides 838 to 843 : PstI restriction site inserted downstream to the stop codon of trpR
Nucleotides 844 to 1468 : sequence of the E. coli genome downstream to the tnaA coding sequence

CTGTCAGATGCGCTTCGCTTCATTGTTACCGCTCCTGTTATTCCTCAACCCTTTTTTTAAACATTAAAATTCTTA
CGTAATTTATAATCTTTAAAAAAAGCATTTAATATTGCTCCCCGAACGATTGTGATTCGATTCACATTTAAACAA
TTTCAGAATAGACAAAAACTCTGAGTGTAATAATGTAGCCTCGTGTCTTGCGAGGATAAGTGCATTATGAATATC
TTACATATATGTGTGACCTCAAAATGGTTCAATATTGACAACAAAATTGTCGATCACCGCCCTTGATTTGCCCTT
CTGTAGCCATCACCAGAGCCAAACCGATTGATTCAATGTGTTCTATTTGTTTGCTATATCTTAATTTTGCCTTTT
GCAAAGGTCATCTCTCGTTTATTTACTTGTTTTAGTAAATGATGGTGCTTGCATATATATCTGGCGAATTAATCG
GTATAGCAGATGTAATATTCACAGGGATCACTGTAATTAAAATAAATGAAGGATTATGTA**ATG**GCCCAACAATCA
CCCTATTCAGCAGCGATGGCAGAACAGCGTCACCAGGAGTGGTTACGTTTTGTCGACCTGCTTAAGAATGCCTAC
CAAAACGATCTCCATTTACCGTTGTTAAACCTGATGCTGACGCCAGATGAGCGCGAAGCGTTGGGGACTCGCGTG
CGTATTGTCGAAGAGCTGTTGCGCGGCGAAATGAGCCAGCGTGAGTTAAAAAATGAACTCGGCGCAGGCATCGCG
ACGATTACGCGTGGATCTAACAGCCTGAAAGCCGCGCCCGTCGAGCTGCGCCAGTGGCTGGAAGAGGTGTTGCTG
AAAAGCGAT**TGACTGCAG**TTAATACTACAGAGTGGCTATAAGGATGTTAGCCACTCTCTTACCCTACATCCTCAA
TAACAAAAATAGCCTTCCTCTAAAGGTGGCATCATGACTGATCAAGCTGAAAAAAAGCACTCTGCATTTTGGGGT
GTTATGGTTATAGCAGGTACAGTAATTGGTGGAGGTATGTTTGCTTTACCTGTTGATCTTGCCGGTGCCTGGTTT
TTCTGGGGTGCCTTTATCCTTATCATTGCCTGGTTTTCAATGCTTCATTCCGGGTTATTGTTATTAGAAGCAAAT
TTAAATTATCCCGTCGGCTCCAGTTTTAACACCATCACCAAAGATTTAATCGGTAACACCTGGAACATTATCAGC
GGTATTACCGTTGCCTTCGTTCTCTATATCCTCACTTATGCCTATATCTCTGCTAATGGTGCGATCATTAGTGAA
ACGATATCAATGAATTTGGGTTATCACGCTAATCCACGTATTGTCGGGATCTGCACAGCCATTTTCGTTGCCAGC
GTATTGTGGTTAAGTTCGTTAGCCGCCAGTCGTATTACCTCATTGTTCCTCGGGCTGAAGATTATCTCCTTTGTG
ATCGTGTTTGGTTCTTTTTTCTTCCAGGTCGATTACTCCATTC